

## Identification of Bacterial Groups Preferentially Associated with Mycorrhizal Roots of *Medicago truncatula*<sup>▽</sup>

P. Offre,<sup>1</sup> B. Pivato,<sup>1,2</sup> S. Siblot,<sup>1</sup> E. Gamalero,<sup>2</sup> T. Corberand,<sup>1</sup> P. Lemanceau,<sup>1</sup> and C. Mougel<sup>1\*</sup>

INRA-Université de Bourgogne, UMR Microbiologie et Géochimie des Sols, CMSE, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France,<sup>1</sup>  
and Università del Piemonte Orientale Amedeo Avogadro, Dipartimento di Scienze dell'Ambiente e della Vita,  
via Bellini 25/G, 15100 Alessandria, Italy<sup>2</sup>

Received 29 August 2006/Accepted 20 November 2006

The genetic structures of bacterial communities associated with *Medicago truncatula* Gaertn. cv. Jemalong line J5 (Myc<sup>+</sup> Nod<sup>+</sup>) and its symbiosis-defective mutants TRV48 (Myc<sup>+</sup> Nod<sup>-</sup>) and TRV25 (Myc<sup>-</sup> Nod<sup>-</sup>) were compared. Plants were cultivated in a fertile soil (Châteaurenard, France) and in soil from the Mediterranean basin showing a low fertility (Mas d'Imbert, France). Plant growth, root architecture, and the efficiency of root symbiosis of the three plant genotypes were characterized in the two soils. Structures of the bacterial communities were assessed by automated-ribosomal intergenic spacer analysis (A-RISA) fingerprinting from DNA extracted from the rhizosphere soil and root tissues. As expected, the TRV25 mutant did not develop endo-mycorrhizal symbiosis in any of the soils, whereas mycorrhization of line J5 and the TRV48 mutant occurred in both soils but at a higher intensity in the Mas d'Imbert (low fertility) than in the Châteaurenard soil. However, modifications of plant growth and root architecture, between mycorrhizal (J5 and TRV48) and nonmycorrhizal (TRV25) plants, were recorded only when cultivated in the Mas d'Imbert soil. Similarly, the genetic structures of bacterial communities associated with mycorrhizal and nonmycorrhizal plants differed significantly in the Mas d'Imbert soil but not in the Châteaurenard soil. Multivariate analysis of the patterns allowed the identification of molecular markers, explaining these differences, and markers were further sequenced. Molecular marker analysis allowed the delineation of 211 operational taxonomic units. Some of those belonging to the *Comamonadaceae* and *Oxalobacteraceae* ( $\beta$ -*Proteobacteria*) families were found to be significantly more represented within bacterial communities associated with the J5 line and the TRV48 mutant than within those associated with the TRV25 mutant, indicating that these bacterial genera were preferentially associated with mycorrhizal roots in the Mas d'Imbert soil.

Mycorrhizae result from symbiotic associations between soil fungi and the roots of most plants. Mycorrhizae are considered to be classic examples of mutualistic symbioses. The basis for this mutualism relies on the supply of carbon to the fungus by the host plant and, in return, on the supply of mineral nutrients and water to the plant and on the plant's protection against soil-borne diseases by the fungus (41). Among mycorrhizal symbioses, arbuscular mycorrhizae (AM) are the most widespread. AM are recorded in 80% of land plants and are generated by the association of plant roots and fungal populations belonging to the *Glomeromycota* phylum (37, 41), which includes around 160 species (23, 45). AM are ancient; the first fossil evidence of this symbiosis dates back 400 million years (33). Several authors have proposed that AM have contributed to the colonization of early land plants (32, 38).

AM are generally assumed to be nonspecific associations, since *Glomeromycota* are able to colonize roots of several host plants and are themselves colonized by different AM fungal species (12, 14, 35, 44). Despite this lack of host specificity, the diversity of AM fungi has been shown to affect the plant community composition under field conditions (43), and the genetic structure of the AM fungal community was shown to

differ significantly according to the plant species (B. Pivato, P. Lemanceau, S. Siblot, G. Berta, C. Mougel, and D. van Tuinen, submitted for publication). The long, joint evolution of plants and AM fungi is expected not to have occurred independently of the resident bacteri flora. However, very few studies have addressed the relationship between free-living bacteria and AM fungi. Meyer and Linderman (21) have reported changes in the bacteri flora associated with *Trifolium subterraneum* mycorrhized with *Glomus fasciculatum* and in that associated with *Zea mays* mycorrhized with *Glomus fasciculatum*, the former showing a higher density of facultative anaerobic bacteria and the latter a lower density of fluorescent pseudomonads and chitinase-producing actinomycetes. These results contrast with the report of Andrade et al. (3) indicating that the mycorrhization of *Sorghum bicolor* by *Glomus etunicatum*, *G. intraradices*, or *G. mosseae* led to an increased density of pseudomonads. Following an untargeted approach, Marschner et al. (19) and Artursson et al. (5), having used denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism analysis, have stressed that changes occur in the genetic structures of bacterial communities associated with roots of maize and wheat, respectively, when they are colonized by AM fungus inoculation. However, none of these studies has led to an identification of taxa that explains these variations.

Overall, information on bacteria preferentially associated with mycorrhizal roots appears scarce. Furthermore, the corresponding studies did not take into account the natural diver-

\* Corresponding author. Mailing address: INRA-Université de Bourgogne, UMR Microbiologie et Géochimie des Sols, CMSE, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France. Phone: 33-3 8069 3053. Fax: 33-3 8069 3224. E-mail: mougel@dijon.inra.fr.

<sup>▽</sup> Published ahead of print on 1 December 2006.

sity of AM fungi, since they were based on the inoculation of selected strains of *Glomeromycota*. However, under field conditions, plants are generally colonized by several types of functionally different *Glomeromycota* (12, 13, 14, 44; Pivato et al., submitted). Therefore, the inoculation of specific strains of *Glomeromycota* to assess their impact on the indigenous bacterioflora may have induced bias. To avoid this bias, an option previously proposed relies on removing indigenous AM fungi in soil to get nonmycorrhizal control treatment (31). For that purpose, fungicide applications have been applied (26); however, again, these treatments are likely to induce bias by affecting the soil microflora, especially the fungi.

In the 1990s, Sagan et al. (34) obtained mutants of *Medicago truncatula* that are affected in their ability to develop symbiotic associations. In this study, we proposed using these mutants to assess the impact of AM established between this model plant and indigenous populations of *Glomeromycota* on bacterial communities in the rhizosphere.

The aim of this study was to compare the genetic structures of rhizosphere bacterial communities associated with mycorrhizal and nonmycorrhizal genotypes of *M. truncatula* (34) using an automated-ribosomal intergenic spacer analysis (A-RISA) for DNA fingerprinting (30). The corresponding experiments were performed in soil with a low level of fertility from the Mediterranean basin, corresponding to the diversification zone of annual medics (the Mas d'Imbert region, France), and in fertile soil which never supported medic growth (Châteaurenard, France). Molecular markers revealing variations in the genetic structure of the bacterial communities were identified and sequenced in order to determine the genera preferentially associated with mycorrhizal roots.

#### MATERIALS AND METHODS

**Experimental design.** The experiments were conducted with *Medicago truncatula* Gaertn. cv. Jemalong line J5 (Myc<sup>+</sup> Nod<sup>+</sup>) and its symbiosis-defective mutants TRV48 (Myc<sup>+</sup> Nod<sup>-</sup>) and TRV25 (Myc<sup>-</sup> Nod<sup>-</sup>) (34). Seeds of the three genotypes were scarified and surface sterilized by gently shaking them in 98% sulfuric acid for 2 min, 95% ethanol for 5 min, and 3.5% sodium hypochlorite solution for 10 min, and rinsed successively six times for 5 min in sterile demineralized water. Seeds were germinated on 0.7% (wt/vol) water agar plates at 25°C for 48 h. One germinated seed was sown per cylindrical polyvinylchloride container (diameter, 9 cm; height, 20 cm) filled with 1.5 liter of a sandy-clay loam soil (Châteaurenard, France) or with a silt-clay loam soil (Mas d'Imbert, France), corresponding to 1,380 g or 1,560 g, respectively. Soil characteristics are summarized in Table 1. According to agronomic standards (22), the amount of mineral phosphorus in the Châteaurenard soil was appropriate for plant growth but was limiting in the Mas d'Imbert soil. Eighteen containers per soil type were used for each *M. truncatula* genotype. Three additional containers without plant (bulk soil) were used as controls for each soil type.

Plants were cultivated in a growth chamber in a 16:8-h light/dark photoperiod in a 23:18°C light/dark thermoperiod, with photosynthetically active radiation at 500  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  at pot height and 55% relative humidity. Water was added each day to maintain humidity at 55% of the water holding capacity. Containers were randomly distributed in the growth chamber and moved each day. The plants were sampled after 34 days, corresponding to the appearance of a shoot ramification of order 2 (24).

**Quantification of plant growth and characterization of root architecture.** Plant growth was quantified based on the root and shoot dry weight (DW) of four plant replicates per experimental treatment, after drying the fresh material at 80°C for 48 h.

Root architecture was characterized based on the same plants by evaluating six morphometric parameters: total root length, total root surface area, total root volume, mean root diameter, the number of root tips, and root branching degree (represented by root tip number divided by total root length) using a dedicated

TABLE 1. Physicochemical characteristics of the soils used in the present study

Characteristic	Soil	
	Châteaurenard	Mas d'Imbert
Clay (%)	17.20	39.40
Silt (%)	47.40	49.10
Sand (%)	35.40	11.50
pH	7.90	7.60
NO <sub>3</sub> <sup>-</sup> (mg/kg)	14.30	2.60
NH <sub>4</sub> <sup>+</sup> (mg/kg)	1.30	0.42
P <sub>2</sub> O <sub>5</sub> (g/kg)	0.46	0.03
Total organic matter (g/kg)	26.20	23.00
C/N ratio	13.74	12.18

scanner and WhinRhizo Pro software (Réagents Instrument Inc., Québec, Canada) as previously described (11).

**Evaluation of plant nodulation and mycorrhization.** Evaluation of plant nodulation and mycorrhization was performed on five plant replicates per experimental treatment. The level of nodulation was assessed by enumerating the nodules per root system and by weighting them after desiccation at 80°C for 48 h.

Visualization of total AM fungi was made with trypan blue staining of 30 root pieces, about 1 cm long, randomly sampled from the whole root system of each plant (27). Quantification of arbuscular mycorrhizal infection and colonization was performed using the notation scale described by Trouvelot et al. (42) and expressed as the frequency of root infection, the colonization intensity of the root cortex, the colonization intensity of the mycorrhizal root cortex, the abundance of arbuscules in the root cortex, and the abundance of arbuscules in the mycorrhizal root cortex. Parameters of mycorrhization were calculated with MYCO-CALC software, available at <http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html>.

**DNA extraction from soils and roots.** DNA was extracted from bulk soils, rhizosphere soils, and root tissues. Three replicates were analyzed per experimental treatment. Each bulk soil replicate was made of 1 gram of dry bulk soil sampled per container after homogenization of the soil. Each root replicate was made of three root systems with adhering soil sampled from three different containers. Rhizosphere soil was obtained from roots by shaking the three root systems twice in a flask containing 100 ml of distilled sterile water at 200 rpm for 10 min (Agitest; Bioblock Scientific, Illkirch, France). The root systems were removed from the soil suspension, and the rhizosphere soil was collected after centrifugation at 8,000  $\times g$  for 10 min. These root systems were ground with a mortar and pestle in liquid nitrogen to get the root tissue compartment. Each sample was frozen in liquid nitrogen and conserved at -80°C for further use in DNA extraction.

DNA extraction, purification, and quantification procedures were performed as previously described by Ranjard et al. (30). Briefly, 1 gram of dry bulk soil, rhizosphere soil, or root tissues was shaken with glass beads and a lysis solution containing sodium dodecyl sulfate in a bead beater. The samples were centrifuged, and the supernatants were collected. Proteins and cell fragments were precipitated with 5 M potassium acetate and discarded after centrifugation. DNA was then precipitated from the supernatants with isopropanol and centrifuged. The pellet of DNA was washed with ethanol and dissolved in Tris-EDTA buffer. DNA was separated from residual impurities, and particularly humic substances, by centrifugation through two types of minicolumns. DNA extracts were loaded onto PVPP (polyvinylpyrrolidone) microcolumns (Bio-Rad, Marne la Coquette, France) and eluted by centrifugation. The eluates were collected and then purified a second time using a GENECLEAN Turbo nucleic acid purification kit (QBiogene, Illkirch, France) following the manufacturer's instructions.

The concentrations of purified DNA extracts were quantified by comparing them to standard curves of calf thymus DNA concentrations. Purified DNA samples as well as calf thymus DNA dilutions (Bio-Rad, Marne la Coquette, France) were resolved by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide and photographed (Biocapt; Vilber Lourmat, Marne la Vallée, France). The intensity of the ethidium bromide fluorescence was integrated with ImageQuant software (Molecular Dynamics, Evry, France).

**Automated-RISA fingerprinting.** The bacterial ribosomal intergenic spacers (IGSs) were amplified with the primers S-D-Bact-1522-b-S-20 (5'-TGCGGC TGGATCCCTCCTT-3') and L-D-Bact-132-a-A-18 (5'-CCGGGTTTCCCATTCGG-3') (25). PCR conditions, PCR template preparation for DNA

TABLE 2. Quantification of plant growth and root morphometric parameters<sup>a</sup>

Soil	Plant genotype	Dry wt			Root				No. of root tips	Root branching degree
		Shoot (g)	Root (g)	Root/shoot ratio	Total length (cm)	Total surface area (cm <sup>2</sup> )	Total vol (cm <sup>3</sup> )	Mean diam (mm)		
Châteaurenard	Myc <sup>+</sup> Nod <sup>+</sup>	1.48 a	0.67 a	0.45 b	6,875.74 a	1,063.42 a	13.17 a	0.49 c	6,454.25 a	0.93 a
	Myc <sup>+</sup> Nod <sup>-</sup>	1.33 b	0.48 b	0.36 b	6,593.69 a	987.34 a	11.87 a	0.48 c	5,830.25 a	0.88 a
	Myc <sup>-</sup> Nod <sup>-</sup>	0.61 c	0.36 b	0.59 b	5,328.87 b	828.96 b	10.36 a	0.49 c	4,923.00 a	0.92 a
Mas d'Imbert	Myc <sup>+</sup> Nod <sup>+</sup>	0.31 d	0.23 c	0.73 b	1,913.83 c	537.79 c	12.08 a	0.88 a	1,330.50 b	0.69 a
	Myc <sup>+</sup> Nod <sup>-</sup>	0.32 d	0.19 c	0.60 b	1,831.98 c	510.19 c	11.35 a	0.88 a	1,479.75 b	0.81 a
	Myc <sup>-</sup> Nod <sup>-</sup>	0.03 e	0.04 d	1.29 a	556.00 d	104.11 d	1.55 b	0.59 b	318.75 c	0.56 a

<sup>a</sup> Quantification of plant growth and root morphometric parameters of *Medicago truncatula* Gaertn. cv. Jemalong J5 (Myc<sup>+</sup> Nod<sup>+</sup>) and its symbiosis-defective mutants TRV48 (Myc<sup>+</sup> Nod<sup>-</sup>) and TRV25 (Myc<sup>-</sup> Nod<sup>-</sup>) cultivated for 34 days in Châteaurenard or Mas d'Imbert soil. Mean values with the same letter are not significantly different according to the Student-Newman-Keul multiple range test.

sequencer loading, and electrophoresis conditions were those described by Ranjard et al. (30).

A modified A-RISA based on the amplification of IGS plus a contiguous fragment of 16S rRNA gene (467 bp) was performed in order to characterize the bacterial diversity. 16S IGS sequences were amplified by PCR from soil and root DNA extracts with the primers 1055F (5'-ATGGCTGTCGTCAGCTT-3') and L-D-Bact-132-a-A-18. PCR conditions were the same as those used in A-RISA fingerprinting except for the number of amplification cycles, which was increased from 25 to 30 cycles.

**Cloning and sequencing of A-RISA markers.** A-RISA markers explaining the differences between genetic structures of the bacterial communities associated with mycorrhizal and nonmycorrhizal plants were cloned and sequenced in order to identify the corresponding bacterial groups. Identification of these groups was made possible by cloning and sequencing 467 bp of the 16S rRNA gene with the contiguous bacterial ribosomal IGS (36).

PCR products were then separated by an overnight electrophoresis at 40 V in a 1.25% (wt/vol) agarose gel (20 by 25 cm) in 0.1 mM Na<sub>2</sub>EDTA Tris-acetate-EDTA (TAE) buffer. Gel sections containing discriminating markers were excised from 16S-IGS fingerprints of bacterial communities associated with mycorrhizal *M. truncatula*. DNA was eluted from the gel using a DNA Gel Extraction kit (Millipore, Bedford, MA). The eluted DNA was purified and concentrated with a MinElute PCR purification kit (QIAGEN GmbH, Hilden, Germany). The purified DNA was ligated into pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. Ligation products were electroporated into *Escherichia coli* DH10B ElectroMAX cells (Invitrogen SARL, Cergy Pontoise, France), and a blue/white screening was used to identify transformants containing inserts. For each gel section, 96 transformants, making one clone library, were subjected to single-colony isolation and cryopreserved at -80°C in 40% glycerol.

Inserts were directly amplified by PCR from 5 µl of a LB culture of each transformant by using universal primers SP6 (5'-TATTTAGGTGACACTATA G-3')/T7 (5'-TAATACGACTCACTATAGGG-3'). PCR conditions included a first step at 94°C for 5 min to lyse bacterial cells, followed by 30 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. The final extension step was at 72°C for 5 min. PCR products were checked using 2% (wt/vol) agarose gels for insert size control and were sequenced using the 1055F primer. Sequencing was performed by the GENOME express company with a standard sequencing protocol. Sequences were compared to those found in the GenBank database (8) using the BLAST algorithm (2). Sequences showing at least 98% similarity were grouped as an operational taxonomic unit (OTU).

**Statistical analysis.** Plant growth, root architecture, nodulation, and mycorrhization data were submitted to variance analysis ( $P = 0.05$ ), followed by the multiple range test of Student-Newman-Keuls using Statview software (SAS Institute Inc., Cary, NC). Ratios of wild-type DW (shoots and roots) versus TRV25 mutant DW (shoots and roots) were submitted to variance analysis ( $P = 0.05$ ), followed by the multiple range test of Student-Newman-Keuls. Mycorrhization data, expressed as percentages, were submitted to angular transformation prior to statistical analysis. Genetic structures of bacterial communities were compared using principal component analysis, as reported by Ranjard et al. (29). Encoded bands of DNA fingerprints were also plotted in two dimensions based on the scores for the first two principal components in order to point A-RISA markers explaining the differences between the fingerprints of bacterial communities. Sequence inventories of 16S IGS clone libraries generated from rhizosphere soil and root tissues of *M. truncatula* wild-type and symbiosis-defective mutants were compared with an exact  $P$  value chi-square test. Most significant

sequences were identified by performing an analysis of contingency table cell contribution to the chi-square value. Chi-square tests were performed with StatXact 3 (CYTEL Software Corporation, Cambridge, United Kingdom).

## RESULTS

**Plant growth and root architecture characterization.** Plant growth expressed as root and shoot DW of all *M. truncatula* genotypes was significantly higher in the fertile soil of Châteaurenard than in the Mas d'Imbert soil (Table 2). The differentials of growth between the wild-type and the symbiosis-defective mutants differed also in the two soils. The reduced DWs of the shoot and root in the mutant Myc<sup>-</sup> Nod<sup>-</sup> compared to those of the wild type were more significant ( $P < 0.0001$ ) in the Mas d'Imbert soil (10 and 5 times, respectively) than in the Châteaurenard soil (2.4 and 2.2 times, respectively). The reduced growth of the Myc<sup>+</sup> Nod<sup>-</sup> mutant compared to that of the wild type was significant only in the Châteaurenard soil. In this soil, the root DWs of the Myc<sup>+</sup> Nod<sup>-</sup> and Myc<sup>-</sup> Nod<sup>-</sup> mutants did not differ significantly, indicating that the reduced root growth of the mutants was related to their inability to nodulate rather than their inability to mycorrhize; in contrast, the fact that the shoot DW was significantly lower in the Myc<sup>-</sup> Nod<sup>-</sup> mutant than in the Myc<sup>+</sup> Nod<sup>-</sup> mutant indicates that both symbionts contributed to the shoot growth. The root/shoot DW ratio did not differ significantly among the different experimental conditions, except for the Myc<sup>-</sup> Nod<sup>-</sup> mutant, which had the significantly highest ratio.

The values of root morphometric parameters (total root length, total root surface, total root volume, mean root diameter, number of root tips, and root branching degree) are shown in Table 2. These parameters were significantly highest in the Châteaurenard soil, except for the mean root diameter, which was higher in the Mas d'Imbert soil, and for the root branching degree, which did not differ significantly between the two soils. Total root length and root surface of the Myc<sup>-</sup> Nod<sup>-</sup> mutant were significantly reduced compared to those of the wild type and the Myc<sup>+</sup> Nod<sup>-</sup> mutant in both soils. Total root volume, root diameter, and number of root tips of the Myc<sup>-</sup> Nod<sup>-</sup> mutant were significantly reduced compared to those of the two other plant genotypes only in the Mas d'Imbert soil.

**Nodulation.** The nodulation rate was quantified for all *M. truncatula* genotypes by evaluating the number and DW of nodules (Table 3). As expected, nodules were detected only for the wild-type genotype. Their number and total dry weight

TABLE 3. Quantification of nodulation and mycorrhization parameters<sup>a</sup>

Soil	Plant genotype	Nodulation		Mycorrhization <sup>b</sup>				
		Nodule no.	Nodule DW (mg)	F%	M%	m%	A%	a%
Châteaurenard	Myc <sup>+</sup> Nod <sup>+</sup>	195 b	9.4 b	92 b	48 b	53 b	23 b	47 b
	Myc <sup>+</sup> Nod <sup>-</sup>	0	0	94 b	39 b	42 b	22 b	57 a
Mas d'Imbert	Myc <sup>+</sup> Nod <sup>+</sup>	351 a	21.8 a	100 a	76 a	76 a	46 a	61 a
	Myc <sup>+</sup> Nod <sup>-</sup>	0	0	97 a	70 a	73 a	40 a	56 a

<sup>a</sup> Quantification of nodulation and mycorrhization parameters of *Medicago truncatula* Gaertn. cv. Jemalong J5 (Myc<sup>+</sup> Nod<sup>+</sup>) and TRV48 (Myc<sup>+</sup> Nod<sup>-</sup>) cultivated for 34 days in Châteaurenard and Mas d'Imbert soils. Mean values with the same letter are not significantly different according to the Student-Newman-Keuls multiple range test.

<sup>b</sup> Mycorrhization parameters were submitted to the angular transformation of percentage values prior performing the statistical analyses. Mycorrhization parameters are frequency of root infection (F%), colonization intensity of the root cortex (M%), colonization intensity of the mycorrhized root cortex (m%), abundance of arbuscules in the root cortex (A%), and abundance of arbuscules in the mycorrhized root cortex (a%).

were significantly higher for plants grown in the Mas d'Imbert soil than in the Châteaurenard soil.

**Mycorrhization.** The level of the mycorrhizal colonization was assessed for each plant genotype by quantifying the frequency and the colonization intensity of the root cortex, the colonization intensity of the mycorrhizal root cortex, the arbuscular abundance in the root cortex, and the arbuscular abundance in the mycorrhizal root cortex (Table 3). Appressorium development was observed for all plant genotypes in the two soils, and as expected, infection of the root cortex occurred only in the wild type (J5) and the Myc<sup>+</sup> Nod<sup>-</sup> mutant (TRV48). For these plant genotypes, all mycorrhization parameters were significantly higher in the Mas d'Imbert soil than in the Châteaurenard soil. Mycorrhization parameters did not differ significantly between mycorrhizal genotypes (J5 and TRV48) in both soils, except for the rate of arbuscules, which was significantly higher in the Myc<sup>+</sup> Nod<sup>-</sup> mutant than in the wild type when cultivated in the Châteaurenard soil.

**DNA fingerprints of bacterial communities.** DNA fingerprints of bacterial communities provided complex profiles for all compartments (bulk soil, rhizosphere soil, and root tissues) in the Châteaurenard and the Mas d'Imbert soils (Fig. 1A and B, respectively). In the Châteaurenard soil, A-RISA markers ranged from 250 bp (100-bp IGS) to 1,051 bp (901-bp IGS) for bulk soil and rhizosphere soils, and from 250 bp (100-bp IGS) to 1,336 bp (1,186-bp IGS) for root tissues. In the Mas d'Imbert soil, A-RISA bands ranged from 230 bp (80-bp IGS) to 1,120 bp (970-bp IGS) for bulk soil and rhizosphere soils, and from 250 bp (100-bp IGS) to 1,414 bp (1,264-bp IGS) for root tissues.

Visual observations of A-RISA gels indicated that for a given experimental condition, minor variations were detected among replicates (none of the bands differed between replicates, but their relative intensities may have sometimes differed).

**Effect of plant genotypes on the genetic structure of bacterial communities.** The effect of plant genotypes on the genetic structures of bacterial communities in the rhizosphere soil and root tissues was characterized with principal component analysis (Fig. 2). The genetic structures of bacterial communities of the root tissues differed significantly from those of the rhizosphere soil, which differed from those of the bulk soil communities

(data not shown). The genetic structures of bacterial communities associated with a given plant genotype in both compartments (rhizosphere soils and root tissues) always differed significantly from those of the bulk soils (data not shown).

In the Châteaurenard soil, the genetic structures of bacterial communities from the rhizosphere soils of the three plant genotypes were similar (Fig. 2A). The three rhizosphere soils were not separated on the principal component plot based on the first two principal components which explained 43% and 24% of total variability, respectively (Fig. 2A). In contrast, the genetic structures of bacterial communities from the root tissues of the wild type (J5) differed significantly from those of the symbiosis-defective mutants (TRV48 and TRV25) (Fig. 2B). Bacterial communities from the root tissues of the wild-type line were separated from those of both mutants according to the first principal component which explained 38% of total variability (Fig. 2B). As shown by a two-dimensional plot of the encoded markers (data not shown), these differences were mostly related to two molecular markers of 1,316 bp and 1,356 bp (IGS sizes of 1,166 bp and 1,206 bp, respectively) which were present in A-RISA fingerprints from the nodulated wild type but absent in those from the two Nod<sup>-</sup> mutants (Fig. 1A), the two markers being in the size range of  $\alpha$ -Proteobacteria and more specifically rhizobia (28). Bacterial communities from the root tissues of the symbiosis-defective mutants TRV48 and TRV25 were not separated on the principal component plot (Fig. 2B), indicating that they did not differ significantly.

In the Mas d'Imbert soil, the genetic structures of the bacterial communities from the rhizosphere soils of the wild type (J5) and the Myc<sup>-</sup> Nod<sup>-</sup> mutant (TRV25) were significantly different (Fig. 2C), but those of the Myc<sup>+</sup> Nod<sup>-</sup> mutant (TRV48) did not differ significantly from those of J5 and of the TRV25 mutant. The communities from the rhizosphere soils of the wild type and the Myc<sup>-</sup> Nod<sup>-</sup> mutant (TRV25) were separated on the first principal component, which explained 69% of the total variability (Fig. 2C), whereas those from the rhizosphere soil of the Myc<sup>+</sup> Nod<sup>-</sup> mutant (TRV48) were less clearly separated from the wild type. According to the two-dimensional plots of A-RISA markers, differences between the rhizosphere soils of J5 and TRV25 were mainly explained by three markers of 340, 350, and 400 bp (IGS sizes of 190, 200, and 250 bp, respectively) localized in zone I as indicated in Fig. 1B (data not shown). These markers were present in all fingerprints but expressed with a greater relative intensity in those of the mycorrhizal genotypes (J5 and TRV48). The genetic structures of bacterial communities from the root tissues of the mycorrhizal genotypes (J5 and TRV48) differed significantly from those of the nonmycorrhizal genotype (TRV25), whereas bacterial communities of mycorrhizal genotypes did not differ significantly (Fig. 2D). Communities from the root tissues of the mycorrhizal and the nonmycorrhizal genotypes were separated on the second principal component, which explained 23% of the variability (Fig. 2D). According to the two-dimensional plot of A-RISA markers (data not shown), differences between the genetic structure of the mycorrhizal and that of the nonmycorrhizal genotype were explained mostly by (i) a molecular marker of 1,300 bp (IGS size of 1,150 bp) (Fig. 1B); (ii) a group of three markers of 710, 730, and 750 bp (IGS sizes of 560, 570, and 580 bp, respectively) localized in zone II, as indicated in Fig. 1B; and (iii) a group of three markers of 460,

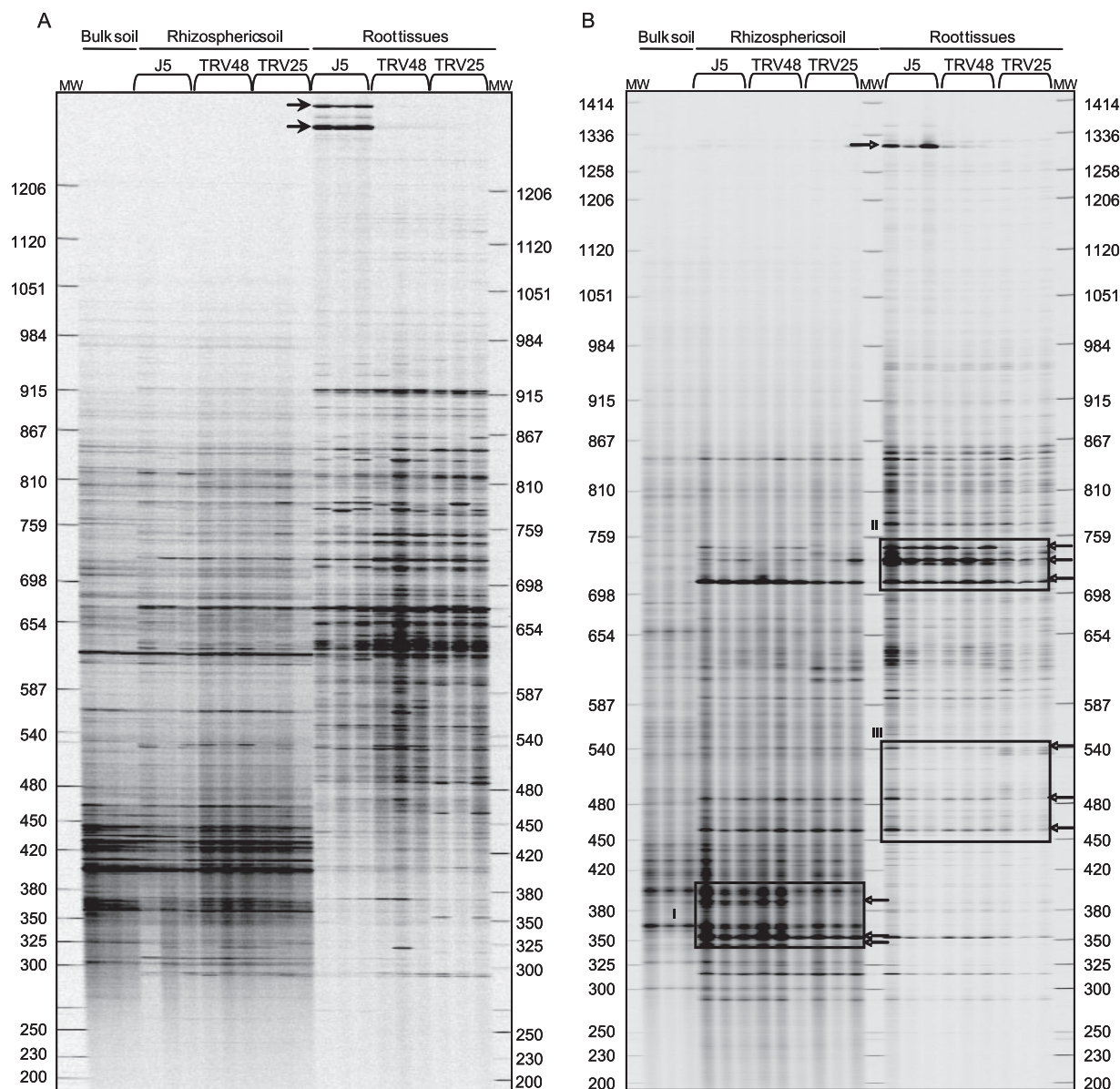


FIG. 1. A-RISA DNA fingerprints of bacterial communities from bulk soil, rhizosphere soils, and root tissues of *Medicago truncatula* Gaertn. cv. Jemalong line J5 ( $\text{Myc}^+ \text{Nod}^+$ ) and its symbiosis-defective mutants TRV48 ( $\text{Myc}^+ \text{Nod}^-$ ) and TRV25 ( $\text{Myc}^- \text{Nod}^-$ ) cultivated in Châteaurenard (A) and Mas d'Imbert (B) soils. Arrows indicate A-RISA markers explaining differences between communities of the three genotypes. Outlined gel sections show evidence zones, including markers preferentially associated with both mycorrhizal genotypes of *M. truncatula*. MW, molecular weight.

480, and 540 bp (IGS sizes of 310, 330, and 390 bp, respectively) localized in zone III, as indicated in Fig. 1B. The 1,300-bp marker was present only in the fingerprint of the wild type, whereas the markers from zones II and III were present in both types of fingerprints but expressed with greater relative intensities in those from the mycorrhizal genotypes.

Markers only present or expressed with a greater relative intensity in the DNA fingerprints from the mycorrhizal genotypes were considered as being preferentially associated with these genotypes.

**Diversity analysis of A-RISA markers preferentially associated with mycorrhizal genotypes of *M. truncatula*.** Bacterial

groups corresponding to A-RISA markers preferentially associated with mycorrhizal genotypes (J5 and TRV48) were identified by coupling A-RISA with diversity analysis of cloned 16S IGS sequences.

Fingerprints obtained from modified (16S IGS) and regular A-RISA (IGS) differed only by the size increase of the bands related to the additional 16S rRNA gene fragment amplified in the modified A-RISA. Principal component analysis of both types of fingerprints gave similar results in terms of ordination of the different genetic structures of bacterial communities (data not shown).

Ninety-six sequences from zones I, II and III, as shown in

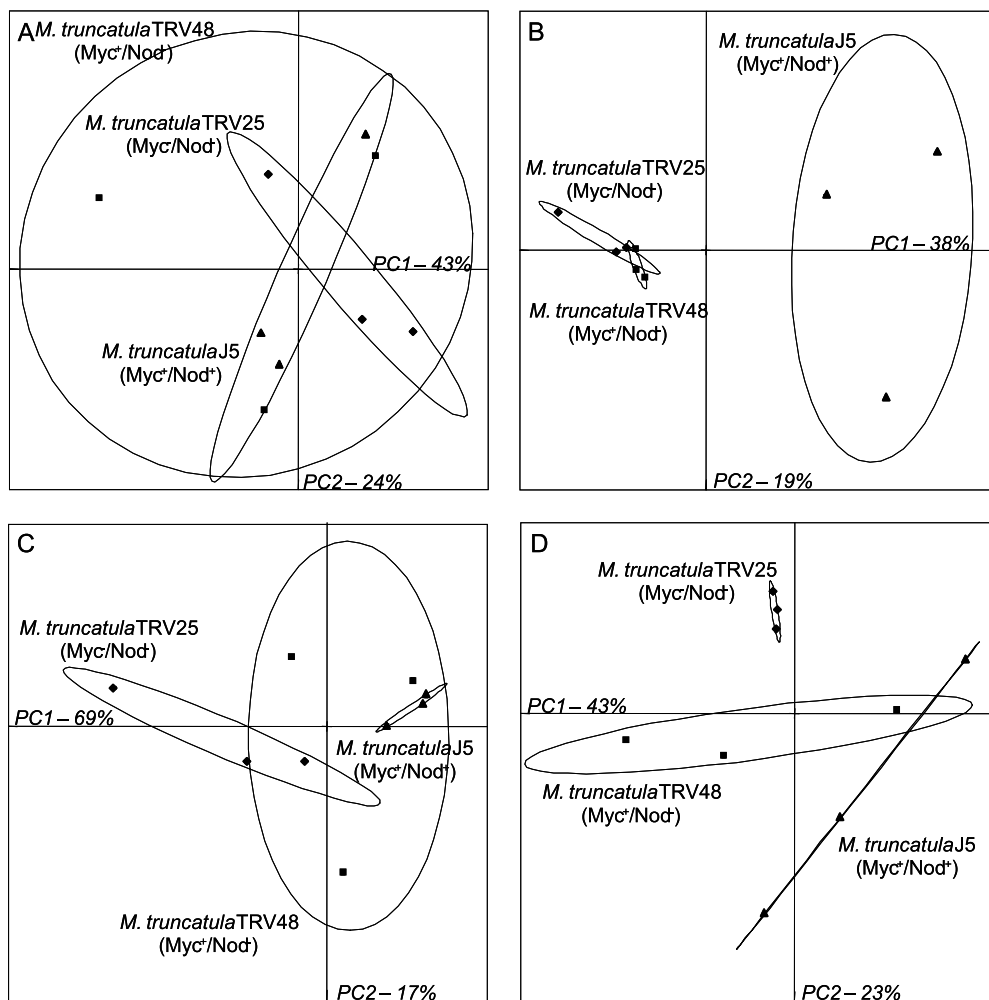


FIG. 2. Comparison of the genetic structures of bacterial communities of *Medicago truncatula* Gaertn. cv. Jemalong line J5 ( $\text{Myc}^+ \text{Nod}^+$ ) ( $\blacktriangle$ ) and its symbiosis-defective mutants TRV48 ( $\text{Myc}^+ \text{Nod}^-$ ) ( $\blacksquare$ ) and TRV25 ( $\text{Myc}^- \text{Nod}^-$ ) ( $\blacklozenge$ ) by principal component A-RISA profiles obtained from the different compartments: (A) rhizosphere soil of plants grown in the Châteaurenard soil; (B) root tissues of plants grown in the Châteaurenard soil; (C) rhizosphere soil of plants grown in the Mas d'Imbert soil; and (D) root tissues of plants grown in the Mas d'Imbert soil. Ellipses represent 90% confidence limits.

Fig. 1B, were shown to include markers preferentially associated with the mycorrhizal genotypes were analyzed with BLAST for each plant genotype. The frequency distributions of OTUs from the corresponding clone libraries differed significantly between the mycorrhizal and the nonmycorrhizal genotypes ( $\chi^2$ , exact  $P$  value  $< 0.05$ ). These differences were explained mostly by variations of the frequency of two OTUs common to the three plant genotypes (cell contributions to the  $\chi^2$  value) (Table 4). The 16S rRNA gene fragment of the most represented OTU in zone I (22%, 31%, and 15% clone in libraries of J5, TRV48, and TRV25, respectively) and zone III (70%, 76%, and 47%, respectively) showed 99% similarity with that of *Collimonas fungivorans* (GenBank accession number 33145982). The 16S rRNA gene fragments of the most represented OTUs in zone II (42%, 56%, and 17%, and 23%, 9%, and 6%) showed 97 and 99% similarity with those of *Methylobium petroleiphilum* (GenBank accession number 6063088) and *C. fungivorans* (GenBank accession number 33145982), respectively. Most (80%) of the remaining OTUs were repre-

sented only by one clone, and altogether they corresponded to less than 5% of the analyzed clones. The frequency distributions of OTUs associated with the mycorrhizal genotypes (J5 and TRV48) did not differ significantly ( $\chi^2$ , exact  $P$  value  $> 0.05$ ).

## DISCUSSION

The aims of this work were to determine whether AM affect the bacterial communities and whether bacterial groups appear to be preferentially associated with AM. The strategy consisted of comparing the genetic structures of the indigenous bacterial communities associated with *M. truncatula* Gaertn. cv. Jemalong line J5, which is known to establish symbiotic associations with AM fungi and rhizobia ( $\text{Myc}^+ \text{Nod}^+$ ), together with its symbiosis-defective mutants TRV48 ( $\text{Myc}^+ \text{Nod}^-$ ) and TRV25 ( $\text{Myc}^- \text{Nod}^-$ ) (34). In contrast to previous studies (5, 19), our strategy avoided the inoculation of AM fungal isolates and allowed taking into account the diversity of

TABLE 4. Frequency and identity of the closest relatives of the most abundant OTUs<sup>a</sup>

Clone library	Size range of IGS sequences (bp)	Compartment	No. of OTUs in clone libraries	Frequency of the most abundant OTUs	Closest relative in GenBank of the most abundant OTUs (accession no.)	% of similarity	GenBank accession no. of the most abundant OTUs
Zone I							
J5 (Myc <sup>+</sup> Nod <sup>+</sup> )	135–285	Rhizosphere soil	42	21 <sup>b</sup> (22) <sup>c</sup>	<i>Collimonas fungivorans</i> (33145982)	99	AM396920
TRV48 (Myc <sup>+</sup> Nod <sup>−</sup> )			44	30 (31)	<i>Collimonas fungivorans</i> (33145982)	99	AM396921
TRV25 (Myc <sup>−</sup> Nod <sup>−</sup> )			50	14 (15)	<i>Collimonas fungivorans</i> (33145982)	99	AM396922
Zone II							
J5 (Myc <sup>+</sup> Nod <sup>+</sup> )	485–635	Root tissues	24	40 (42)	<i>Methylibium petroleiphilum</i> (6063088)	97	AM396923
				22 (23)	<i>Collimonas fungivorans</i> (33145982)	99	AM396926
TRV48 (Myc <sup>+</sup> Nod <sup>−</sup> )			24	54 (56)	<i>Methylibium petroleiphilum</i> (6063088)	97	AM396924
				9 (9)	<i>Collimonas fungivorans</i> (33145982)	99	AM396927
TRV25 (Myc <sup>+</sup> Nod <sup>−</sup> )			46	16 (17)	<i>Methylibium petroleiphilum</i> (6063088)	97	AM396925
				6 (6)	<i>Collimonas fungivorans</i> (33145982)	99	AM396928
Zone III							
J5 (Myc <sup>+</sup> Nod <sup>+</sup> )	285–435	Root tissues	24	67 (70)	<i>Collimonas fungivorans</i> (33145982)	99	AM396929
TRV48 (Myc <sup>+</sup> Nod <sup>−</sup> )			16	73 (76)	<i>Collimonas fungivorans</i> (33145982)	99	AM396930
TRV25 (Myc <sup>−</sup> Nod <sup>−</sup> )			25	45 (47)	<i>Collimonas fungivorans</i> (33145982)	99	AM396931

<sup>a</sup> Frequency and identity of the closest relatives of the most abundant OTUs, from the zones defined in Fig. 1 as those including A-RISA markers preferentially associated with *Medicago truncatula* Gaertn. cv. Jemalong J5 (Myc<sup>+</sup> Nod<sup>+</sup>) and TRV48 (Myc<sup>+</sup> Nod<sup>−</sup>).

<sup>b</sup> Number of clones of the most abundant OTUs.

<sup>c</sup> Percentage of clones of the most abundant OTUs out of the total number of clones.

indigenous AM fungi establishing symbiotic associations with *M. truncatula* in the two soils tested.

Plants were cultivated in a soil from the Mediterranean area corresponding to the diversification zone of annual medics (Mas d'Imbert, France), showing a low fertility level, and in a more fertile soil from the green belt of Châteaurenard (France) (Table 1). The genetic structures of bacterial communities were assessed by A-RISA DNA fingerprinting (24) in the two corresponding bulk soils and in the rhizosphere soils and root tissues of the three plant genotypes cultivated in these soils (Fig. 1). A-RISA DNA fingerprinting was previously shown to be sensitive and robust enough to detect spatiotemporal changes in the genetic structures of the bacterial communities in the rhizosphere of *M. truncatula* (24).

In all soils and plant genotypes, the genetic structure of the bacterial communities associated with the roots differed significantly from those of the corresponding bulk soils, these differences being more strongly expressed in the root tissues than in the rhizosphere soils (data not shown). These data are in agreement with our previous work (24) and underline the gradient of the rhizosphere effect.

The genetic structures of the bacterial communities were shown to differ significantly between (i) the *M. truncatula* wild type (J5) and its Myc<sup>+</sup> Nod<sup>−</sup> mutant (TRV48) and (ii) the Myc<sup>−</sup> Nod<sup>−</sup> mutant (TRV25) when cultivated in the Mas d'Imbert soil but not in the Châteaurenard soil (Fig. 2). Since *M. truncatula* wild type (J5) and its Myc<sup>+</sup> Nod<sup>−</sup> mutant (TRV48) were well mycorrhized in both soils and since, as expected, AM establishment was aborted in the Myc<sup>−</sup> Nod<sup>−</sup> mutant (TRV25) (Table 3), variations in the structures of bacterial communities recorded between (i) J5 and TRV48 and (ii) TRV25 could then be related to AM symbiosis. Taken together, our data allow the conclusion that AM had a structuring effect on the bacterial communities in the Mediterranean fallow soil but not in the fertile soil. In agreement with the spatial gradient of the rhizosphere effect, this impact was

more strongly expressed in the root tissues than in the rhizosphere soil.

In contrast, nodulation appeared to have no significant structuring effect on the bacterial communities in the Mas d'Imbert soil. Indeed, their genetic structures did not differ significantly between the *M. truncatula* wild type (J5) and its Myc<sup>+</sup> Nod<sup>−</sup> mutant (TRV48), either in the rhizosphere soil or in the root tissues. Nodulation had a structuring effect on communities of nodulating bacteria but had no structuring effect on communities of free-living bacteria, as indicated by the fact that differences between J5 and TRV48 communities were explained mostly by molecular markers representing rhizobia. The structuring effect of rhizobia on bacterial communities from the Châteaurenard soil but not from the Mas d'Imbert soil is consistent with the lack of positive effect of nodulation on plant growth in the Mas d'Imbert soil in contrast with that of Châteaurenard, probably related to other limiting factors than nitrogen in the fallow soil of the Mas d'Imbert.

The structuring effect of AM on the bacterial communities in the Mediterranean fallow soil and its absence in the Châteaurenard soil could be ascribed to their origins and characteristics. The Mas d'Imbert soil was sampled in a Mediterranean area in which medics occur endemically (39). Medics have evolved in this area for a very long period of time together with soil microflora (7). Soils from the Mediterranean zone, including those of Mas d'Imbert, are then expected to harbor AM fungi but also bacteria associated with these fungi that are adapted to medic species, especially *M. truncatula*. The diversity of AM fungi in this soil appeared indeed to be high (Pivato et al., submitted). In contrast to the Mas d'Imbert soil, which has a long joint history with medics, the Châteaurenard soil is an alluvial soil in which no medics have been recorded to occur spontaneously or to be cultivated. The absence of bacterial groups preferentially associated with mycorrhizal roots in the soil of Châteaurenard could then result from the fact that these groups were not present among the indigenous populations.

This is in agreement with the significant differences recorded between the genetic structure and the diversity of the bacterial communities associated with the two uncultivated soils (data not shown). This hypothesis of coevolution in centers of host diversification between plants and associated soil-borne microorganisms has recently been proposed when analyzing *Rhizobium etli* diversity and its symbiosis with *Phaseolus vulgaris* (1).

Besides their origins, the two soils also differ in their levels of fertility. The Mas d'Imbert soil is a fallow soil, whereas the Châteaurenard soil is fertile and cultivated with vegetable and fruit trees. This difference of fertility, especially the higher phosphorus content in the Châteaurenard than in the Mas d'Imbert soil (Table 1), may have accounted for the significantly lower rate of mycorrhization in the former than in the latter. The lower fertility of the Mas d'Imbert soil together with the higher mycorrhization rate of the *Myc*<sup>+</sup> genotypes (Table 3) have led to a more significantly beneficial effect of AM symbiosis on plant growth and development in the Mas d'Imbert than in the Châteaurenard soil (Table 2). Since root exudates are influenced quantitatively and qualitatively by the plant growth and development (15, 16, 17, 20), the more significant plant growth promotion by AM in the Mas d'Imbert soil than in the Châteaurenard soil is expected to have impacted more root exudation of the wild type compared to the nonmycorrhizal mutants in the Mas d'Imbert soil than in the Châteaurenard soil. Root exudates are known to influence the genetic structure and diversity of microbial communities in the rhizosphere (18, 40), and differences between the bacterial communities associated with mycorrhizal and nonmycorrhizal genotypes in the two soils could then be related to root exudate variations resulting from the greater promoting effect of AM in the Mas d'Imbert than in the Châteaurenard soil (Table 2). This hypothesis is supported by the observation made by Azaïzeh et al. (6) showing that under optimal growth conditions, which is the case in the fertile soil of Châteaurenard, mycorrhizal and nonmycorrhizal plants release similar amounts of total carbohydrates, reducing sugars, amino acids, phenolics, and organic acids.

Since variations of the genetic structure of bacterial communities according to the AM symbiotic status were only recorded in the Mas d'Imbert soil (Fig. 2), identification of bacterial groups preferentially associated with AM was performed in plants cultivated in this soil. Three groups of molecular markers, preferentially associated with the mycorrhizal genotypes J5 and TRV48, explaining the differences between bacterial communities from the mycorrhizal and nonmycorrhizal *M. truncatula*, were cloned and sequenced (Fig. 1B). Analysis of the frequency of the most representative sequences confirmed that the corresponding markers were preferentially associated with the mycorrhizal genotypes (Table 4). The 16S rRNA gene fragment of the corresponding OTUs presented 99 and 97% similarity with that of *C. fungivorans* and *M. petroleiphilum*. *C. fungivorans* was previously described as a chitinolytic bacterium and to grow at the expense of different soil fungi in soil (9). These species differ from those described by Artursson and Jansson (4) as being preferentially associated with AM fungi, but their study was made directly in a fallow soil and not on roots.

To our knowledge, the present study is the first to assess the impact of AM symbiosis on the indigenous bacterial commu-

nities and to identify the groups that appear to be preferentially associated with AM. These groups appear to belong to the *Burkholderiales* order. The next step of our research will be to assess the effects that these bacterial groups have on AM symbiosis. From an evolutionary point of view, one may expect that some effects are beneficial to mycorrhization, as it was shown with a fluorescent pseudomonad representative of populations preferentially associated with ectomycorrhizae (10); however, other effects could take advantage of the symbiosis as suggested by the presence of chitinolytic bacteria described here to be preferentially associated with AM.

## ACKNOWLEDGMENTS

This study was supported by programs funded by INRA Santé des Plantes et Environnement (Rhizosphere Ecology of Annual Medics program) and the Burgundy regional project (02511CPO2S188) and a doctoral fellowship to P. Offre.

We are grateful to G. Duc (URLEG-INRA, Dijon, France) for providing seeds of *M. truncatula*, D. Pouhair for cultivation of the plants, and N. Chevrier for technical assistance. We thank G. Ganesh for support during this work.

## REFERENCES

1. Aguilar, O. M., O. Rival, and E. Peltzer. 2004. Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification. *Proc. Natl. Acad. Sci. USA* **101**:13548–13553.
2. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
3. Andrade, G., K. L. Mihara, R. G. Linderman, and G. J. Bethlenfalvay. 1997. Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. *Plant Soil* **192**:71–79.
4. Artursson, V., and J. K. Jansson. 2003. Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. *Appl. Environ. Microbiol.* **69**:6208–6215.
5. Artursson, V., R. D. Finlay, and J. K. Jansson. 2005. Combined bromodeoxyuridine immunocapture and terminal-restriction fragment length polymorphism analysis highlights differences in the active soil bacterial metagenome due to *Glomus mosseae* inoculation or plant species. *Environ. Microbiol.* **7**:1952–1966.
6. Azaïzeh, H. A., H. Marschner, V. Römhild, and L. Wittenmayer. 1995. Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth, mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza* **5**:321–327.
7. Béna, G., A. Lyet, T. Huguet, and I. Olivieri. 2005. *Medicago-Sinorhizobium* symbiotic specificity evolution and the geographic expansion of *Medicago*. *J. Evol. Biol.* **18**:1547–1558.
8. Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and D. L. Wheeler. 2005. GenBank. *Nucleic Acids Res.* **33**:D34–D38.
9. De Boer, W., J. H. L. Leveau, G. A. Kowalchuk, P. J. A. Klein Gunnewiek, E. C. A. Abeln, M. J. Figge, K. Sjölema, J. D. Janse, and J. A. van Veen. 2004. *Collimonas fungivorans* gen. nov., sp. nov. a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *Int. J. Syst. Evol. Microbiol.* **54**:857–864.
10. Frey, P., P. Frey-Klett, J. Garbaye, O. Berge, and T. Heulin. 1997. Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with Douglas fir *Laccaria bicolor* mycorrhizosphere. *Appl. Environ. Microbiol.* **63**:1852–1860.
11. Gamalero, E., M. G. Martinotti, A. Trotta, P. Lemanceau, and G. Berta. 2002. Morphogenetic modifications induced by *Pseudomonas fluorescens* A6RI and *Glomus mosseae* BEG12 in the root system of tomato differ according to plant growth conditions. *New Phytol.* **155**:293–300.
12. Gollotte, A., D. van Tuinen, and D. Atkinson. 2004. Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaries* and *Lolium perenne* in a field experiment. *Mycorrhiza* **14**:111–117.
13. Helgason, T., A. H. Fitter, and J. P. W. Young. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. *Mol. Ecol.* **8**:658–666.
14. Helgason, T., J. W. Merryweather, J. Denison, P. Wilson, J. P. W. Young, and A. H. Fitter. 2002. Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. *J. Ecol.* **90**:371–384.
15. Hütsch, B. W., J. Augustin, and W. Merbach. 2002. Plant rhizodeposition—an important source for carbon turnover in soils. *J. Plant Nutr. Soil Sci.* **165**:397–407.
16. Jones, D. L., A. Hodge, and Y. Kuzyakov. 2004. Plant and mycorrhizal regulation of rhizodeposition. *New Phytol.* **163**:459–480.

17. **Krafczyk, I., G. Trollenier, and H. Beringer.** 1984. Soluble root exudates of maize: influence of potassium supply and rhizosphere microorganisms. *Soil Biol. Biochem.* **16**:315–322.
18. **Marilley, J., and M. Aragno.** 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Appl. Soil Ecol.* **13**:127–136.
19. **Marschner, P., D. E. Crowley, and R. Lieberei.** 2001. Arbuscular mycorrhizal infection changes the bacterial 16 S rDNA community composition in the rhizosphere of maize. *Mycorrhiza* **11**:297–302.
20. **Martin, J. K.** 1977. Factors influencing the loss of organic carbon from wheat roots. *Soil Biol. Biochem.* **9**:1–7.
21. **Meyer, J. R., and R. G. Linderman.** 1986. Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. *Soil Biol. Biochem.* **18**:191–196.
22. **Morel, C., C. Plenchette, and J. C. Fardeau.** 1992. La fertilisation phosphate raisonnée de la culture du blé. *Agronomie* **12**:565–579.
23. **Morton, J. B., and G. L. Benny.** 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* **37**:471–491.
24. **Mougel, C., P. Offre, L. Ranjard, T. Corberand, E. Gamalero, C. Robin, and P. Lemanceau.** 2006. Dynamic of the genetic structure of bacterial and fungal communities at different development stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5. *New Phytol.* **170**:165–175.
25. **Normand, P., C. Ponsonnet, X. Nesme, M. Neyra, and P. Simonet.** 1996. ITS analysis of prokaryotes, p. 1–12. *In* D. L. Akkermans, J. D. van Elsas, and E. I. de Bruijn (ed.), *Molecular microbial ecology manual*. Kluwer Academic Publishers, Amsterdam, The Netherlands.
26. **Paul, N. D., P. G. Aryes, and L. E. Wyness.** 1989. On the use of fungicides for experimentation in natural vegetation. *Funct. Ecol.* **3**:759–769.
27. **Phillips, J. M., and D. S. Hayman.** 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **55**:158–161.
28. **Ranjard, L., E. Brothier, and S. Nazaret.** 2000. Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl. Environ. Microbiol.* **66**:5334–5339.
29. **Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbière, J. Thioulouse, and S. Nazaret.** 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA). *Microb. Ecol.* **39**:263–272.
30. **Ranjard, L., D. P. H. Lejon, C. Mougel, L. Scherer, D. Merdinoglu, and R. Chaussod.** 2003. Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. *Environ. Microbiol.* **5**:1111–1120.
31. **Read, D. J.** 2002. An ecological point of view on arbuscular mycorrhiza research, p. 129–136. *In* S. Gianinazzi, H. Schüepp, J. M. Barea, and K. Haselwandter (ed.), *Mycorrhizal technology in agriculture. From genes to bioproducts*. Birkhäuser Verlag, Basel, Switzerland.
32. **Redecker, D., R. Kodner, and L. E. Graham.** 2000. Glomalean fungi from the Ordovician. *Science* **289**:1920–1921.
33. **Remy, W., T. N. Taylor, H. Hass, and H. Kerp.** 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proc. Natl. Acad. Sci. USA* **91**:11841–11843.
34. **Sagan, M., D. Morandi, E. Tarengi, and G. Duc.** 1995. Selection of nodulation and mycorrhizal mutants in the model plant *Medicago truncatula* (Gaertn.) after  $\gamma$ -ray mutagenesis. *Plant Sci.* **111**:63–71.
35. **Sanders, I. R.** 2002. Specificity in the arbuscular mycorrhizal symbiosis, p. 415–437. *In* M. G. A. van der Heijden, and I. R. Sanders (ed.), *Mycorrhizal ecology*. Springer, Berlin, Germany.
36. **Schloss, P. D., A. G. Hay, D. B. Wilson, and L. P. Walker.** 2003. Tracking temporal changes of bacterial community fingerprints during the initial stages of composting. *FEMS Microbiol. Ecol.* **46**:1–9.
37. **Schüssler, A., D. Schwarzott, and C. Walker.** 2001. A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycol. Res.* **105**:1413–1421.
38. **Selosse, M.-A., and F. Le Tacon.** 1998. The land flora: a phototroph-fungus partnership? *Trends Ecol. Evol.* **13**:15–20.
39. **Small, E., and M. Jomphe.** 1989. A synopsis of the genus *Medicago* (Leguminosae). *Can. J. Bot.* **67**:3260–3294.
40. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* **67**:4742–4751.
41. **Smith, S. E., and D. J. Read.** 1997. *Mycorrhizal symbiosis*. Academic Press, London, United Kingdom.
42. **Trouvelot, A., J. L. Kough, and V. Gianinazzi-Pearson.** 1986. Mesure du taux de mycorhization VA d'un système racinaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle, p. 217–221. *In* V. Gianinazzi-Pearson, and S. Gianinazzi (ed.), *Physiological and genetical aspects of mycorrhizae*. INRA, Paris, France.
43. **Van der Heijden, M. G. A., J. N. Klironomos, M. Ursic, P. Moutoglou, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I. R. Sanders.** 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**:69–72.
44. **Van Tuinen, D., E. Jacquot, B. Zhao, A. Gollotte, and V. Gianinazzi-Pearson.** 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Mol. Ecol.* **7**:879–887.
45. **Walker, C., and J. M. Trappe.** 1993. Names and epithets in the Glomales and Endogonales. *Mycol. Res.* **97**:339–344.